Interaction of Picrotoxin with $GABA_A$ Receptor Channel-Lining Residues Probed in Cysteine Mutants

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ABSTRACT We used the substituted-cysteine-accessibility method to identify the channel-lining residues in a region (257–261) near the putative cytoplasmic end of the M2 membrane-spanning segment of the rat γ-aminobutyric acid type A ($GABA_A$) receptor α_1 subunit. The residues α_1Val257 and α_1Thr261 were accessible to charged, sulfhydryl-specific reagents applied extracellularly in both the open and closed states. The accessibility of α_1V257C and α_1T261C in the closed state implies that the gate must be at least as close to the cytoplasmic end of the channel as α_1Val257. Also, the positively charged reagent methanethiosulfonate ethylammonium penetrated from the extracellular end of the channel to α_1T261C, with which it reacted, indicating that the anion-selectivity filter is closer to the cytoplasmic end of the channel than this residue is. Co-application of picrotoxin prevented the sulfhydryl reagents from reacting with α_1V257C but did not prevent reaction with the more extracellular residue α_1T261C. Picrotoxin protection of α_1V257C may be due to steric block by picrotoxin bound in the channel at the level of α_1Val257; however, if this protection is allosteric, it is not due to the induction of the resting closed state in which α_1V257C was accessible to sulfhydryl reagent.

INTRODUCTION

γ-Aminobutyric acid type A ($GABA_A$) receptors, the major inhibitory neurotransmitter receptors in the central nervous system, are members of a ligand-gated ion channel superfamily (Schofield et al., 1987; Wisden and Seeburg, 1992; Cully et al., 1994). The binding of GABA to $GABA_A$ receptors induces the opening of an anion-selective channel that remains open for tens of milliseconds before it closes (Bormann et al., 1987; Amin and Weiss, 1993; Maconochie et al., 1994; Macdonald and Olsen, 1994). The $GABA_A$ receptor binds and is inhibited by epileptogenic agents such as picrotoxin, and cyclodiene insecticides such as dieldrin, (Twyman et al., 1989, 1992; Porter et al., 1992; Macdonald and Olsen, 1994; Nagata et al., 1994). Picrotoxin and the cyclodiene insecticides act as noncompetitive inhibitors of GABA-induced currents (Maksay and Ticku, 1985; ffrench-Constant et al., 1993a,b). The location of the picrotoxin binding site is controversial (Macdonald and Olsen, 1994): Picrotoxin may bind in the channel lumen and act as an open channel blocker (Inoue and Akaike, 1988; Gurley et al., 1995), or picrotoxin may inhibit channel function allosterically (Smart and Constanti, 1986; Newland and Cull-Candy, 1992; Yoon et al., 1993). Consistent with either mechanism is the finding that resistance among insects to cyclodiene insecticides and to picrotoxin is associated with a point mutation of the alanine aligned with rat α_1Val257 to serine (see Fig. 1) (ffrench-Constant et al., 1993a,b). To investigate the mechanism of picrotoxin block of the $GABA_A$ channel, we have applied the substituted-cysteine-accessibility method to a region near the cytoplasmic end of the M2 membrane-spanning segment (257 to 261) of the rat $GABA_A$ α_1 subunit.

Hydropathy analysis of $GABA_A$ receptor subunit sequences predicts that the N-terminal 200–300 amino acids are extracellular; three closely spaced hydrophobic segments (M1, M2, and M3) span the membrane; a loop of variable length is cytoplasmic; a fourth hydrophobic segment (M4) spans the membrane; and the C-terminal segment is extracellular (Olsen and Tobin, 1990; Wisden and Seeburg, 1992). Based on homology with the nicotinic acetylcholine receptor (Karlin, 1993; Unwin, 1993), $GABA_A$ receptors are likely formed by five subunits arranged in a pseudosymmetrical ring around a central ion channel. The ion channel is presumably lined by residues from the membrane-spanning segments.

Using the substituted-cysteine-accessibility method (Akabas et al., 1992, 1994), we previously identified α_1Thr268 and α_1Ile271, two residues near the extracellular end of the $GABA_A$ receptor α_1 subunit M2 membrane-spanning segment, that line the channel (Xu and Akabas, 1993). The substituted-cysteine-accessibility method provides a systematic approach to the identification of residues lining an ion channel (Akabas et al., 1992, 1994). In this method, consecutive residues in putative channel-lining, membrane-spanning segments are mutated to cysteine, one at a time. Each cysteine-substitution mutant is expressed in *Xenopus* oocytes, and if it has near-normal function the susceptibility of the engineered cysteine to chemical modification by charged, sulfhydryl-specific reagents is determined. We assume that only engineered-cysteine residues exposed in the channel will be accessible for modification

Received for publication 16 June 1995 and in final form 14 August 1995.

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0006-3495/95/11/1858/00 $2.00

by the sulfhydryl reagents; those that face the interior of the protein or the lipid bilayer will not be accessible to react with charged, hydrophilic, lipophobic reagents. We infer that for a mutant receptor whose conduction is irreversibly altered by the sulfhydryl-specific reagents, the side chain of the corresponding wild-type residue lines the ion channel.

The sulfhydryl reagents we have used include the negatively charged 4-chloromercuribenzenesulfonate ($pCMBS^-$) and the positively charged methanethiosulfonate-ethylammonium ($CH_3SO_2SCH_2CH_2NH_3^+$) ($MTSEA^+$) (Akabas et al., 1992; Stauffer and Karlin, 1994). $pCMBS^-$ adds a mercuribenzenesulfonate moiety to free sulfhydryls and $MTSEA^+$ adds $-SCH_2CH_2NH_3^+$ to free sulfhydryls to form a mixed disulfide. $MTSEA^+$ is at least 2500 times more soluble in water than in n-octanol (Akabas et al., 1992).

We report that the side chains of the residues α_1Val257 and α_1Thr261 are exposed in the channel in both the open and closed states of the receptor. We infer that the gate and the anion-selectivity filter are located near the cytoplasmic end of the channel. Picrotoxin protects α_1V257C from modification by sulfhydryl reagents but does not protect the more extracellular residue α_1T261C. Finally, we infer that the picrotoxin blocked state is different from the closed state of the channel.

MATERIALS AND METHODS

Oligonucleotide-mediated mutagenesis

The cDNAs encoding the rat α_1 and γ_2 subunits in the pBluescript SK(−) plasmid (Stratagene) were obtained from Dr. P. Seeburg (Shivers et al., 1989; Ymer et al., 1989), and the β_1 subunit in the pBluescript SK vector were obtained from Dr. A. Tobin (Khrestchatisky et al., 1989). The Altered-sites Mutagenesis procedure (Promega) was used to substitute cysteine residues, one at a time, in the α_1 subunit (see Fig. 1). The α_1 cDNA was excised from α_1-pBluescript plasmid by restriction digestion with *Eco*RI and *Kpn*I and ligated into the pAlter-1 plasmid. Following mutagenesis a cassette defined by the restriction enzyme sites for *Nsi*I and *Bst*EII was excised and ligated into wild-type α_1-pBluescript plasmid. Mutations were identified by restriction digestion and confirmed by DNA sequencing.

Preparation of mRNA and oocytes

For in vitro mRNA transcription, the plasmids containing the α_1 and β_1 subunits were linearized with *Hin*dIII and the γ_2 subunit was linearized with *Xba*I. Messenger RNA was synthesized, and oocytes from *Xenopus* laevis were prepared and maintained as described previously (Xu and Akabas, 1993). One day after the oocytes were harvested, they were injected with 50 nl mRNA (200 pg/nl) mixed in the ratio of 1:1:1 (α_1:β_1:γ_2). Experiments were performed 2–5 days after mRNA injection.

Reagents

$MTSEA^+$ was synthesized as described previously (Stauffer and Karlin, 1994) and used at a concentration of 2.5 mM. $pCMBS^-$ was obtained from Sigma (St. Louis, MO) and used at a concentration of 0.5 mM. α-Isopropyl-α-methyl-γ-butyrolactone (αIMGBL) and β-ethyl-β-methyl-γ-butyrolactone (βEMGBL) were synthesized as described previously (Canney et al., 1991). Picrotoxin was obtained from Sigma.

Electrophysiology

GABA-induced currents were recorded from individual oocytes under two-electrode voltage-clamp, at a holding potential of −80 mV. Electrodes were filled with 3 M KCl and had a resistance of <2 megaohms. The ground electrode was connected to the chamber via a 3 M KCl/agar bridge. Data were acquired into a 486/33 MHz computer using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN) and a TL1-DMA data interface (Axon Instruments, Foster City, CA), and software was written using the Axobasic language (Axon Instruments). The oocyte was perfused at 5 ml/min with Ca^{2+}-free Frog Ringer solution (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES, pH 7.5, with NaOH) at room temperature.

Experimental protocol

We tested the susceptibility of wild-type and mutant $GABA_A$ receptors to a 1-min application of the sulfhydryl reagents applied in the presence or absence of 100 μM GABA. The following sequence of perfusing solutions was used: 100 μM GABA (10 s), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min), sulfhydryl reagent ± 100 μM GABA (1 min), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min), and 100 μM GABA (10 s) (see Fig. 2 for example). The average of the two peak currents before the sulfhydryl reagent was applied was compared with the average of the two peak currents after. The fractional effect was taken as $[1 - (I_{GABA,\ after}/I_{GABA,\ before})]$. The sulfhydryl reagents were applied at the following concentrations: 0.5 mM $pCMBS^-$ and 2.5 mM $MTSEA^+$. The sulfhydryl reagents were applied in CFFR solution.

To test the ability of picrotoxin to protect the engineered cysteines from modification by the sulfhydryl reagents, we used the following sequence of perfusion solutions: 100 μM GABA (10 s), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min), 100 μM picrotoxin + 100 μM GABA (1 min), sulfhydryl reagent + 100 μM picrotoxin + 100 μM GABA (1 min), CFFR (3 min), 100 μM GABA (10 s), CFFR (3 min) and 100 μM GABA (10 s), and CFFR (3 min) and 100 μM GABA (10 s) (see Fig. 5 for example). Similar results were obtained in four to six oocytes for the mutants α_1V257C and α_1T261C.

The picrotoxin concentrations that inhibited 50% of the current induced by 100 μM GABA (IC_{50}) and the Hill coefficients for wild type and for the mutants α_1V257C and α_1T261C were determined by sequential co-application of GABA and five concentrations of picrotoxin: 1, 5, 10, 50, and 100 μM. The current was measured after a 20-s co-application of GABA and picrotoxin.

Statistics

Data in Fig. 3 and 4 are presented as the means ± SE. Significance was determined by one-way analysis of variance by the Duncan criteria ($p < 0.05$) using the SPSS-PC statistics program to compare the effect on the mutants with the effect on wild type.

Sequence alignment

The amino acid sequences of various $GABA_A$ receptor subunits in and flanking the M2 membrane-spanning segment were aligned using the pileup routine of the GCG package, version 7 (Genetics Computer Group, Madison, WI).

RESULTS

We mutated, one at a time, five consecutive residues (257 to 261) in the M2 membrane-spanning segment of the rat α_1 subunit to cysteine (Fig. 1) and injected mRNA for the mutant α_1 subunit together with mRNA for wild-type β_1

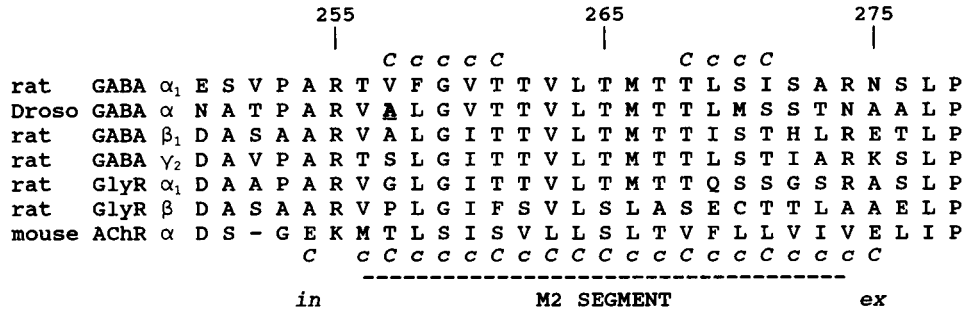


FIGURE 1 The aligned sequences of the amino acid residues (single letter code) in and flanking the M2 membrane-spanning segment of the rat $GABA_A$ receptor α_1, β_1, and γ_2 subunits, the Drosophila $GABA_A$ receptor subunit, the rat glycine receptor α_1 and β subunits, and the mouse muscle acetylcholine receptor α subunit. The upper and lower case italic letters *C* and *c* above and below the aligned sequences indicate residues substituted by cysteine in the $GABA_A$ (this report and Xu and Akabas, 1993) and in the acetylcholine receptors (Akabas et al., 1994). Residues shown to be exposed in the channel are indicated by an upper case *C*; residues not exposed in the channel are indicated by a lower case *c*. The M2 segment is underlined; *in* indicates the intracellular end; *ex* indicates the extracellular end. The numbers at the top indicate the residue number of the $GABA_A$ receptor α_1 subunit. The alanine in bold in the Drosophila sequence is mutated to serine in insects resistant to picrotoxin and cyclodiene insecticides (ffrench-Constant et al., 1993a).

and γ_2 subunits in *Xenopus* oocytes. All of the mutants expressed GABA-induced currents one to three days after injection. We tested the susceptibility of wild-type and mutant $GABA_A$ receptors to irreversible effects of the sulfhydryl reagents by a 1-min application of the reagents in the perfusion solution either in the presence or absence of 100 μM GABA, using two-electrode voltage-clamp recording in intact oocytes (Fig. 2). In the absence of sulfhydryl reagents, the GABA-induced currents were stable over the time course of an experiment. In oocytes expressing wild-type $GABA_A$ receptors, application of either $pCMBS^-$ or $MTSEA^+$, either in the presence or absence of GABA, had no irreversible effects on the GABA-induced currents (Figs. 3 and 4).

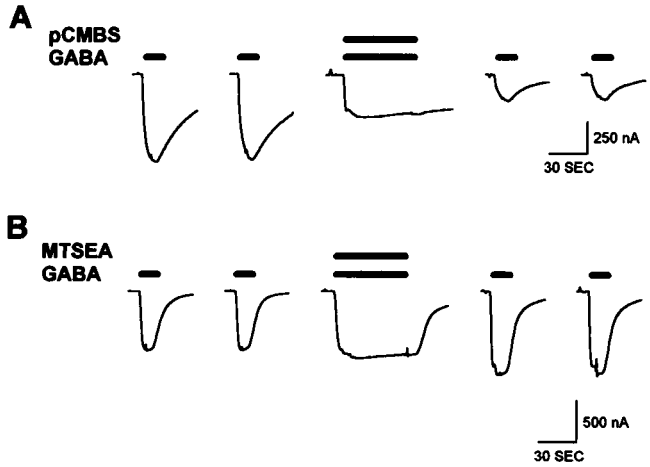


FIGURE 2 The effect of sulfhydryl reagents applied in the presence of GABA on the subsequent GABA-induced currents of two cysteine-substitution mutants. In (*A*) 0.5 mM $pCMBS^-$ is applied to the mutant α_1V257C, and in (*B*) 2.5 mM $MTSEA^+$ is applied to the mutant α_1T261C. The electrical currents recorded by two-electrode voltage clamp from a single oocyte for each mutant are shown. Two responses to 100 μM GABA precede and two responses follow the 1-min application of the sulfhydryl reagent + 100 μM GABA. Each trace is separated by a 3–5 min perfusion with CFFR Ringers. Solution changes are indicated by the black lines above each set of traces.

Effect of $pCMBS^-$ on the cysteine-substitution mutants

In the presence of 100 μM GABA, a 1-min application of 0.5 mM $pCMBS^-$ to the extracellular bath caused significant irreversible inhibition of the mutants α_1V257C and α_1T261C (Figs. 2 *A* and 3 *A*).

In the absence of GABA, in the closed state of the channel, a 1-min application of 0.5 mM $pCMBS^-$ to the extracellular bath caused significant irreversible inhibition of the mutant α_1V257C but had no effect on α_1T261C (Fig. 3 *B*). Application of $pCMBS^-$, in the presence or absence of GABA, had no effect on the mutants α_1F258C, α_1G259C, and α_1V260C (Fig. 3).

Effect of $MTSEA^+$ on the cysteine-substitution mutants

A 1-min application of the cationic $MTSEA^+$ to the extracellular bath in the presence or absence of GABA caused significant irreversible potentiation of the subsequent GABA-induced currents for the mutant α_1T261C but had no effect on the mutant α_1V257C (Fig. 2 *B* and Fig. 4, *A* and *B*). Application of $MTSEA^+$, in the presence or absence of GABA, also had no effect on the other mutants α_1F258C, α_1G259C, and α_1V260C (Fig. 4).

Effect of picrotoxin on the accessibility of the α_1V257C and α_1T261C mutants

The channel-lining residue α_1Val257 aligns with the residue mutated in the Drosophila $GABA_A$ receptor from strains that are resistant to cyclodiene insecticides and picrotoxin (Fig. 1) (ffrench-Constant et al., 1993a). Co-application of

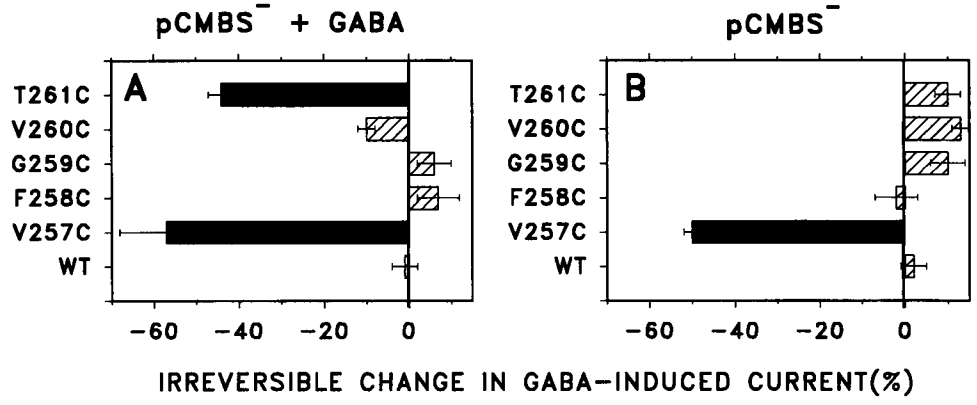


FIGURE 3 The irreversible effect on the GABA-induced currents of wild-type $\alpha_1\beta_1\gamma_2$ and the mutants resulting from a 1-min application of 0.5 mM $pCMBS^-$. (*A*) $pCMBS^-$ applied in the presence of GABA and (*B*) $pCMBS^-$ applied in the absence of GABA. Solid bars indicate mutants for which inhibition was significantly different ($p < 0.05$) than for wild type (*WT*) by one-way analysis of variance. The fractional change was calculated as 1 − (mean final current/mean initial current). Negative change indicates inhibition, and positive change represents potentiation of the GABA-induced currents following application of $pCMBS^-$. The means and SEMs are shown. The number of oocytes for each experimental condition is either three or four.

100 μM picrotoxin with GABA resulted in complete block of the GABA-induced current in oocytes expressing wild-type α_1V257C or α_1T261C (Fig. 5, *A* and *B*). The block was reversible, requiring ~3–5 min for complete restoration of the original GABA-induced current. The picrotoxin concentrations that inhibited 50% of the current induced by 100 μM GABA (IC_{50}) and Hill coefficients were 12.3 ± 4.1 μM, n_H = 1.0 ± 0.07 (n = 4) for wild type; 4.8 ± 2.0 μM, n_H = 0.8 ± 0.05 (n = 4) for α_1T261C; and 4.8 ± 1.0 μM, n_H = 0.7 ± 0.05 (n = 3) for α_1V257C. Thus, the mutations to cysteine caused only small changes in picrotoxin binding as measured by inhibition of macroscopic currents.

We examined whether picrotoxin could protect α_1V257C and α_1T261C from modification by the sulfhydryl reagents. We co-applied GABA and picrotoxin for 1 min to block completely all of the GABA-induced current and then added the sulfhydryl reagent to the perfusion solution with GABA and picrotoxin for an additional 1 min. The GABA, sulfhydryl reagent, and picrotoxin were washed out, and at 3-min intervals the magnitude of the GABA-induced current was tested (Fig. 5 *A*). In oocytes expressing the mutant α_1V257C, the subsequent GABA-induced currents were similar to their initial levels (Figs. 5 *A* and 6 *A*). This indicates that picrotoxin protected α_1V257C from modification by $pCMBS^-$. The extent of protection by picrotoxin was greater when picrotoxin was applied in the presence of GABA as compared with the absence of GABA (Fig. 6 *A*); this is consistent with the use-dependence of the action of picrotoxin (Newland and Cull-Candy, 1992; Yoon et al., 1993).

In oocytes expressing the mutant α_1T261C, co-application of picrotoxin did not protect the sulfhydryl from modification by $MTSEA^+$ (Figs. 5 *B* and 6 *B*) or $pCMBS^-$ (Fig. 6 *C*). Furthermore, the reaction of $MTSEA^+$ with α_1T261C

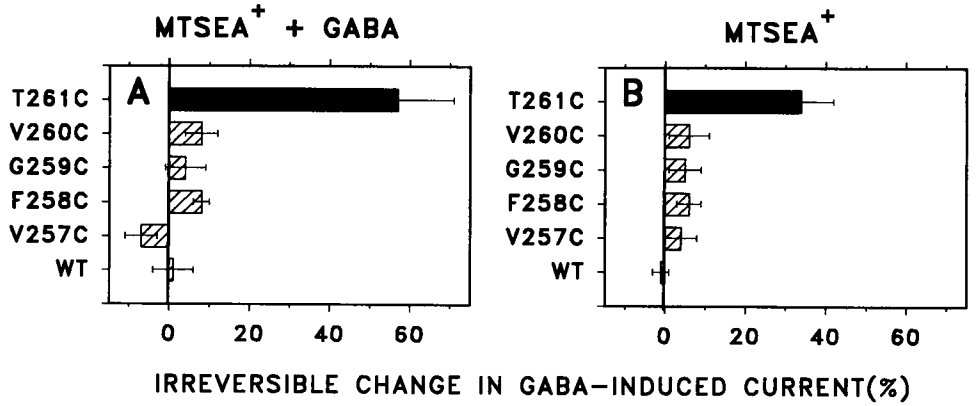


FIGURE 4 The irreversible effect on the GABA-induced currents of wild-type $\alpha_1\beta_1\gamma_2$ and the mutants resulting from a 1-min application of 2.5 mM $MTSEA^+$. (*A*) $MTSEA^+$ applied in the presence of GABA and (*B*) $MTSEA^+$ applied in the absence of GABA. Solid bars indicate mutants for which inhibition was significantly different ($p < 0.05$) than for wild type (WT) by one-way analysis of variance. The fractional change was calculated as 1 − (mean final current/mean initial current). Negative change indicates inhibition, and positive change represents potentiation of the GABA-induced currents following application of $MTSEA^+$. The means and SEMs are shown. The number of oocytes for each experimental condition is between four and six.

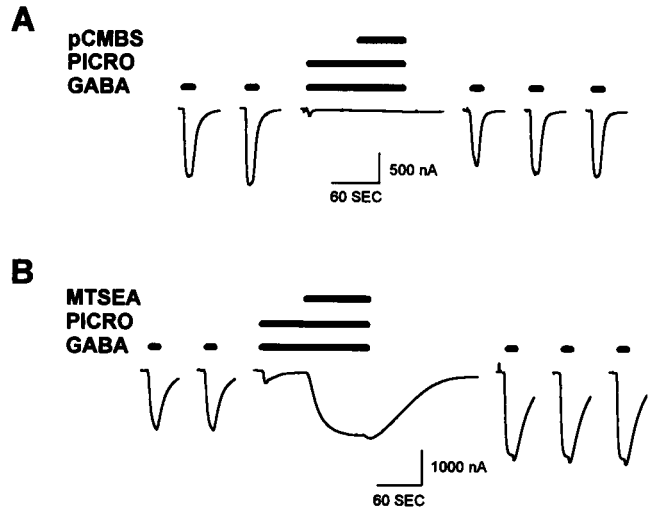


FIGURE 5 The effect of picrotoxin on the ability of the extracellularly applied sulfhydryl reagents to react with engineered cysteines exposed in the channel. Two responses to 100 μM GABA precede and three responses follow the application of sulfhydryl reagent in the presence of 100 μM picrotoxin and 100 μM GABA. In (*A*) 0.5 mM pCMBS is applied to the mutant α_1V257C in the presence of picrotoxin and GABA. The application of picrotoxin in the presence of GABA completely blocks the GABA-induced current before pCMBS is applied. Application of pCMBS has no effect on the subsequent GABA-induced currents. In (*B*) 2.5 mM MTSEA is applied to the mutant α_1T261C in the presence of picrotoxin and GABA. The application of picrotoxin in the presence on GABA completely blocks the GABA-induced current before MTSEA is applied. Note that application of MTSEA results in a large increase in the GABA-induced current even in the continued presence of picrotoxin. The subsequent GABA-induced currents are larger than the initial currents, indicating potentiation of the GABA response. The electrical currents recorded by two-electrode voltage clamp from a single oocyte for each mutant are shown. Each trace is separated by a 3-min perfusion with CFFR Ringers. Solution changes are indicated by the black lines above each set of traces.

in the picrotoxin-blocked state relieved the picrotoxin-induced block (Fig. 5 *B*). Modification of α_1T261C by $MTSEA^+$ increased the IC_{50} for picrotoxin to 25.4 $\pm$ 1.3 μM and reduced the Hill coefficient to 0.47 $\pm$ 0.05 (n = 3).

Effects of γ-butyrolactone derivatives on picrotoxin protection of α_1V257C

The γ-butyrolactone derivative αIMGBL is an antagonist of the action of picrotoxin (Holland et al., 1990; Yoon et al., 1993). αIMGBL (5 mM) had no effect on the current induced by 100 μM GABA (Fig. 7 *A*; compare the magnitude of the initial GABA-induced currents with the initial peak current during the co-application of the reagents). Application of $pCMBS^-$ in the presence of αIMGBL and GABA resulted in irreversible inhibition of the subsequent GABA-induced currents (Fig. 7, *A* and *C*). Thus, αIMGBL by itself did not protect α_1V257C from reaction with $pCMBS^-$.

Co-application of 5 mM αIMGBL, 100 μM picrotoxin, and 100 μM GABA resulted in partial block (~30% reduction) of the GABA-induced current (Fig. 7 *B*; compare the magnitude of the initial GABA-induced currents with the initial peak current during the co-application of the reagents). Application of $pCMBS^-$ in the presence of αIMGBL, picrotoxin, and GABA resulted in irreversible inhibition of the subsequent GABA-induced currents (Fig. 7, *B* and *C*). Thus, αIMGBL blocks the ability of picrotoxin to protect the sulfhydryl from modification.

A second γ-butyrolactone derivative, βEMGBL, partially blocks GABA-induced currents at low concentrations of GABA, but βEMGBL had no effect on currents induced by 100 μM GABA (Yoon et al., 1993). We found that up to 5 mM βEMGBL had no effect on the currents induced by 100 μM GABA and did not protect the sulfhydryl of α_1V257C from reaction with $pCMBS^-$ (data not shown). In the presence of 100 μM GABA, βEMGBL acted as a picrotoxin antagonist; co-application of 5 mM βEMGBL, 100 μM picrotoxin, and 100 μM GABA resulted in only partial block (~30% reduction) of the GABA-induced current (data not shown). Furthermore, application of $pCMBS^-$ in the presence of βEMGBL, picrotoxin, and GABA resulted in irreversible inhibition of the subsequent GABA-induced currents, indicating that the sulfhydryl of α_1V257C was not protected (data not shown). Thus, in the presence of 100 μM GABA, βEMGBL blocked picrotoxin binding, behaving in a manner similar to αIMGBL.

DISCUSSION

Residues exposed in the channel

The GABA-induced responses of two of the cysteine-substitution mutants, α_1V257C and α_1T261C, were irreversibly altered by the sulfhydryl reagents (Figs. 3 and 4). We infer that irreversible alteration of the subsequent GABA-induced responses indicates that the sulfhydryl reagents reacted with an engineered cysteine. We assume that for cysteine residues substituted in the M2 membrane-spanning segment, only those engineered cysteine residues exposed in the channel lumen will be accessible to react with the charged, hydrophilic, sulfhydryl reagents added extracellularly. Furthermore, because the mutants displayed responses to GABA, we assume that the structure of the mutants is similar to the structure of wild type. Therefore, we infer that the side chains of the corresponding wild-type residues α_1Val257 and α_1Thr261 are exposed in the channel. If this region of the M2 membrane-spanning segment is α-helical, then α_1Val257 and α_1Thr261 would lie on the same side of an α-helix about 6 Å apart.

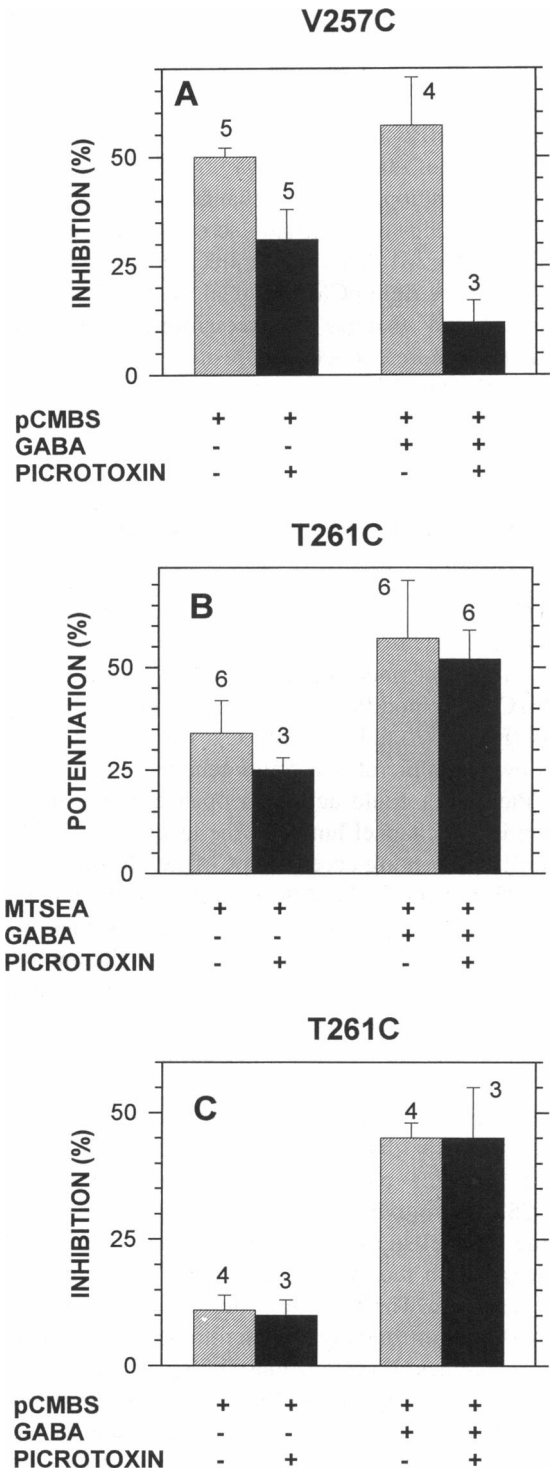
The residues in the α subunit of the nicotinic acetylcholine receptor, Thr244 and Ser248, which align with α_1Val257 and α_1Thr261, are also exposed in the channel lumen (Akabas et al., 1994).

Location of charge-selectivity filter

The $GABA_A$ receptor channel is anion selective; the upper limit of the ratio of potassium permeability relative to

chloride is 0.05 (Bormann et al., 1987). The ability of the cationic reagent, $MTSEA^+$, to penetrate from the extracellular end of the channel to the level of α_1T261C indicates that both anions and cations can enter the extracellular end of the channel. Thus, it is unlikely that the extracellular ring of charged residues identified by Imoto et al., (1988), which aligns with $GABA_A$ α_1Asn275 (Fig. 1), is the only determinant of charge selectivity. Nevertheless, the net positive charge on the extracellular ring would tend to concentrate anions in the mouth of the pore. The charge selectivity filter appears to be at a position that is more cytoplasmic than α_1Thr261; however, the small but potentially finite cation permeability of the $GABA_A$ receptor may account for the ability of $MTSEA^+$ to reach the level of α_1T261C.

The cation-selective acetylcholine receptor displays a similar lack of charge selectivity at its extracellular end. We have previously shown that anionic reagents can enter the acetylcholine receptor channel to the level of αVal255 (Akabas et al., 1994), which aligns with $GABA_A$ receptor residue α_1Thr268 (Fig. 1). The substitution of three residues from the M2 segment of the $GABA_A$ receptor, aligned with α_1 subunit residues Pro253, Ala254, and Thr268, into the aligned positions in the neuronal α7 acetylcholine receptor altered the charge selectivity of the substituted acetylcholine receptor from cation to anion selective (Galzi et al., 1992). Given that cations can penetrate further into the $GABA_A$ channel than α_1Thr268, it seems unlikely that residues at this level in the channel form part of the charge selectivity filter.



Location of the gate and structural changes concomitant with gating

The ability of the sulfhydryl reagents applied in the extracellular bath to react with channel-lining residues in the absence of GABA, i.e., in the closed state of the receptor, implies that the channel lumen is patent in the closed state of the channel from the extracellular end to at least the level of α_1Val257. Furthermore, it implies that the gate, the structure that blocks ion conduction, is located at least as close to the cytoplasmic end of the channel as α_1Val257 is. Given the low membrane permeability of $pCMBS^-$ (Vanstevininck et al., 1965) and its ability to distinguish the sidedness of cysteines in other membrane proteins (Yan and Maloney, 1993), it is unlikely that it could pass through the membrane and enter the channel from the cytoplasmic end to reach α_1Val257 in the closed state; similar arguments can be made for $MTSEA^+$ reaching α_1Thr261. Furthermore, if

FIGURE 6 The effect of picrotoxin on the accessibility of the mutants α_1V257C and α_1T261C to modification by sulfhydryl reagents. (*A*) Picrotoxin protection of the mutant α_1V257C from inhibition by 0.5 mM $pCMBS^-$ applied in the presence or in the absence of 100 μM GABA. Black bars indicate the mean inhibition by $pCMBS^-$ applied in the presence of 100 μM picrotoxin; gray bars indicate the mean inhibition by $pCMBS^-$ applied in the absence of picrotoxin. (*B*) Picrotoxin protection of the mutant α_1T261C from modification by 2.5 mM $MTSEA^+$ applied in the presence or in the absence of 100 μM GABA. Black bars indicate the mean potentiation by $MTSEA^+$ applied in the presence of 100 μM picrotoxin; gray bars indicate the mean potentiation by $MTSEA^+$ applied in the absence of picrotoxin. (*C*) Picrotoxin protection of the mutant α_1T261C from inhibition by 0.5 mM $pCMBS^-$ applied in the presence or in the absence of 100 μM GABA. Black bars indicate the mean inhibition by $pCMBS^-$ applied in the presence of 100 μM picrotoxin; gray bars indicate the mean inhibition by $pCMBS^-$ applied in the absence of picrotoxin. Means ± SEM and the number of oocytes for each case are plotted.

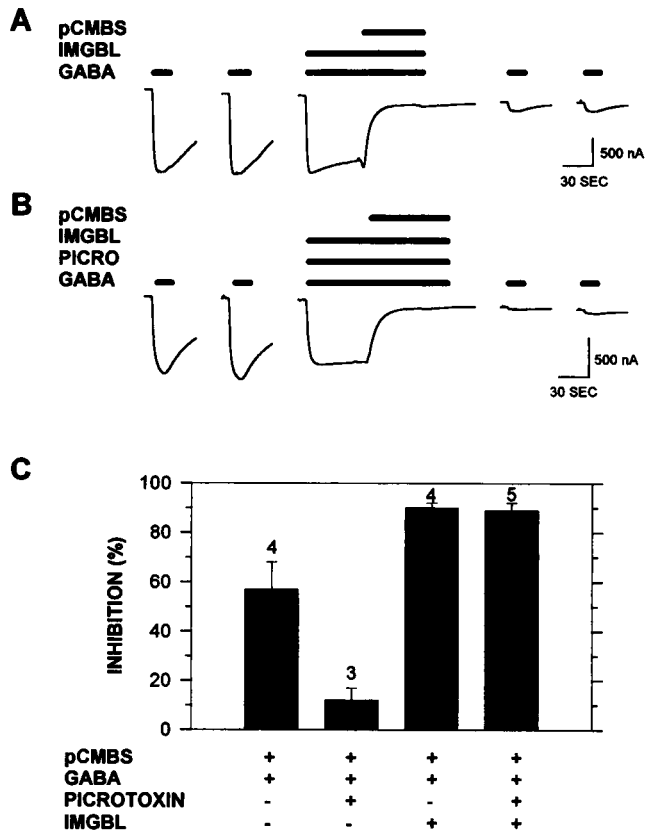


FIGURE 7 The effect of αIMGBL on the accessibility of α_1V257C to reaction with $pCMBS^-$ and protection by picrotoxin. (*A*) The effect of 0.5 mM $pCMBS^-$ applied for 1 min in the presence of 5 mM αIMGBL and 100 μM GABA on the subsequent GABA-induced currents of the α_1V257C mutant. (*B*) The effect of 0.5 mM $pCMBS^-$ applied for 1 min in the presence of 5 mM αIMGBL, 100 μM picrotoxin, and 100 μM GABA on the subsequent GABA-induced currents of the α_1V257C mutant. In (*A*) and (*B*), the electrical currents recorded by two-electrode voltage clamp from single oocytes are shown. Two responses to 100 μM GABA precede and two responses follow the application of reagents + 100 μM GABA. Each trace is separated by a 3–5 min perfusion with CFFR Ringers. Solution changes are indicated by the black lines above each set of traces. (*C*) The average irreversible inhibition of the GABA-induced currents of the mutant α_1V257C resulting from a 1-min application of 0.5 mM $pCMBS^-$ in the presence of various reagents. The left bar indicates that $pCMBS^-$ reacts with the mutant and inhibits subsequent GABA-induced currents. The left center bar indicates that picrotoxin protects the sulfhydryl of the α_1V257C mutant from reacting with $pCMBS^-$. The right center bar indicates that αIMGBL does not protect the sulfhydryl of the α_1V257C mutant from reacting with $pCMBS^-$. The right bar indicates that αIMGBL blocks the ability of picrotoxin to protect the sulfhydryl of α_1V257C. The mean + SEM and number of oocytes tested are indicated for each condition.

$pCMBS^-$ or $MTSEA^+$ had entered the cytoplasm, with its high concentration of free sulfhydryls, the sulfhydryl reagents would most likely have reacted before they could enter the cytoplasmic end of the channel.

Our results showing that the gate is at least as cytoplasmic as α_1Val257 are consistent with the findings of similar experiments in the acetylcholine receptor which imply that the gate is located at a position that is even more cytoplasmic (Akabas et al., 1994). These results identifying the position of the gate are inconsistent with the suggestion that the gate in the acetylcholine receptor is formed by a ring of leucine residues that align with the $GABA_A$ residue α_1Leu264 (Unwin, 1993, 1995).

Although the gate itself is at least as cytoplasmic as or is more cytoplasmic than α_1Val257, the process of gating appears to cause conformational changes in the channel lining at more extracellular positions. In the presence of GABA, both $pCMBS^-$ and $MTSEA^+$ react with α_1T261C, but in the absence of GABA only $MTSEA^+$ reacts with α_1T261C. The susceptibility of α_1T261C to $MTSEA^+$, in both the presence and absence of GABA, suggests that the structural change which reduces the susceptibility of this residue to $pCMBS^-$ in the absence of GABA is not due to the movement of this residue away from the channel lumen to a buried position. Rather a change in the disposition of nearby side chains may partially screen the cysteine substituted for α_1Thr261. This may reduce access more for the larger and more rigid $pCMBS^-$ than for $MTSEA^+$. Similar agonist-induced changes in accessibility have been observed at the more extracellular residue α_1Thr268 (Xu and Akabas, 1993) and for some residues in the M2 segment of the acetylcholine receptor (Akabas et al., 1994).

Site of picrotoxin binding: channel lumen versus allosteric site

Co-application of picrotoxin protects the sulfhydryl of the mutant α_1V257C from modification by $pCMBS^-$ (Figs. 5 *A* and 6 *A*) but does not protect the sulfhydryl of the mutant α_1T261C from modification by the charged, sulfhydryl reagents (Figs. 5 *B* and 6, *B* and *C*). There are two mechanisms by which picrotoxin could generate a nonconducting state. Picrotoxin could act as an open channel blocker by binding in the channel lumen at the level of α_1Val257 and physically preventing access of $pCMBS^-$ to the sulfhydryl of α_1V257C (Fig. 8 *A*). Alternatively, the interaction between picrotoxin and the channel-lining residues may be allosteric, i.e., picrotoxin binds at a distant site and induces a conformational change in the receptor driving it into a nonconducting, blocked state that reduces the accessibility of the sulfhydryl at α_1Val257 but not at α_1Thr261 (Fig. 8 *B*).

The simplest explanation of our results is that picrotoxin acts as a slowly dissociating, open-channel blocker. The binding site for picrotoxin would be at the level of α_1Val257. Picrotoxin would need to penetrate far enough into the channel so as not to obstruct the access of sulfhydryl reagents to the more extracellular, adjacent, channel-lining residue α_1Thr261. The γ-butyrolactones presumably bind in this region and prevent picrotoxin from binding. The γ-butyrolactones, however, must be small enough to bind without blocking the channel and therefore do not protect either α_1V257C or α_1T261C from modification by the sulfhydryl reagents. In addition, because the γ-butyrolactones

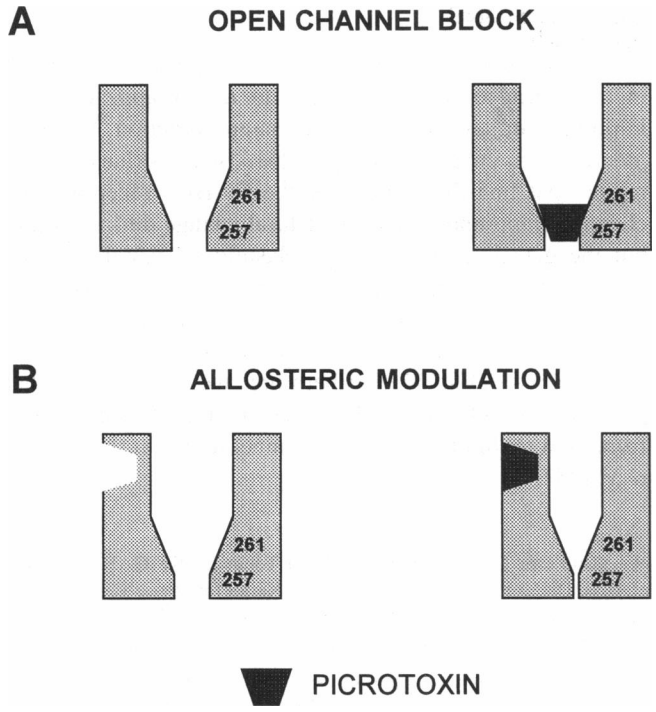


FIGURE 8 Two models for the mechanism of action of picrotoxin are illustrated. (*A*) Illustration of an open channel block mechanism where picrotoxin binds in the channel at the level of α_1Val257 and sterically blocks the channel lumen. (*B*) Illustration of an allosteric mechanism where the binding of picrotoxin at a site distant from the channel lumen induces an occlusion of the pore at the level of α_1Val257.

still act on the α_1V257C and α_1T261C mutants, they probably do not interact strongly with the wild-type residues at either of these positions.

The site at which picrotoxin binds is only accessible from the external side of the membrane because cytoplasmic application of picrotoxin had no effect on GABA-induced currents (Newland and Cull-Candy, 1992). This implies that the picrotoxin binding site is most likely on the water-accessible surface of the protein and that the site is not accessible from the lipid-protein interface, where the sidedness of application should not matter.

If picrotoxin binds in the channel, then the size of picrotoxin and its ability to protect the sulfhydryl at 257 but not at 261 may provide insights into the molecular structure of the channel in this region. Picrotoxinin, the active component of picrotoxin, is a compact, rigid, amphipathic molecule that is roughly spherical with a diameter of ~9 Å, similar to the length of $pCMBS^-$. The channel diameter must be sufficiently large to allow picrotoxin to penetrate from the extracellular end to the level of α_1Val257. In the acetylcholine receptor pore, the narrowest region is thought to be at the level of the residues aligned with α_1Val257 (Villarroel et al., 1991; Cohen et al., 1992). Assuming that the structures of the $GABA_A$ receptor and acetylcholine receptor channels are similar, then the pore diameter must decrease in the region of α_1Val257. On the basis of the relative permeabilities of various sized anions, the "effective pore diameter" of the mouse $GABA_A$ receptor channel at its narrowest point was inferred to be 5.6 Å (Bormann et al., 1987). This diameter is based on the size of the largest permeant anion; if the permeating anion is partially hydrated (a water molecule is ~3 Å in diameter), the actual channel diameter would be larger. Thus, the actual channel diameter may be sufficiently large for picrotoxin, an uncharged amphipathic molecule, to reach the region of α_1Val257.

The residue adjacent to α_1Val257 in the channel-lining, α_1Thr261, influences picrotoxin binding. The sulfhydryl in the α_1T261C mutant is not protected by picrotoxin, and modification by $MTSEA^+$ reduced the affinity for picrotoxin. If the secondary structure of the M2 segment between α_1Val257 and α_1Thr261 is α-helical, then these two residues would lie on the same side of an α-helix approximately 6 Å apart. Given the size of picrotoxin and the estimated separation of these residues, either a chemically modified sulfhydryl at position 261 could interact directly with picrotoxin bound in the region of 257 or the chemical modification of the sulfhydryl at 261 could induce a propagated structural perturbation to the region of the picrotoxin binding site, whether it is at the level of α_1Val257 or at a distant allosteric site. Consistent with these results, mutation of the residues at the positions aligned with α_1Thr261 to phenylalanine in either the α-, β-, or γ subunits abolished picrotoxin sensitivity (Gurley et al., 1995). The fact that the comparable mutation in any subunit inhibited picrotoxin sensitivity is consistent with the picrotoxin binding site being in the channel lumen. Otherwise, one must assume that the mutation in any subunit induces a propagated structural change in all of the subunits to block an allosteric picrotoxin binding site; this seems unlikely because the mutant receptors had normal GABA affinity and benzodiazepine potentiation, suggesting that the mutations had minimal effects on the overall structure of the receptor (Gurley et al., 1995). Furthermore, homomeric glycine receptors formed by expression of the α_1 subunit are sensitive to picrotoxin, but glycine receptors formed by co-expression of the α_1 and β subunits are resistant to picrotoxin (Pribilla et al., 1992). There are several differences in the sequence of the M2 segment of the glycine receptor β subunit that might be responsible for the picrotoxin resistance, notably a proline at the position aligned with α_1Val257 and a phenylalanine at the position aligned with α_1Thr261 (Fig. 1).

Drosophila resistant to cyclodiene insecticides and picrotoxin have a point mutation of alanine to serine at residue 302 in the $GABA_A$ receptor α subunit. This residue aligns with the channel-lining residue Val257 in the rat α_1 subunit (Fig. 1). Picrotoxin inhibits wild-type Drosophila $GABA_A$ receptors with an IC_{50} of ~1 μM; the A302S mutation reduces the picrotoxin sensitivity by 100-fold (ffrench-Constant et al., 1993a). The A302S mutant is also reported to stabilize the open state relative to the desensitized state (Zhang et al., 1994). The presence of a serine at this level in the channel does not prevent picrotoxin binding because

there is a serine in the rat γ_2 subunit at the aligned position (Fig. 1); however, the subunit stoichiometry of the rat and Drosophila $GABA_A$ receptors, and thus the number of serines at this position in the two receptors, is unknown. Nevertheless, the fact that resistance to picrotoxin and insecticide is conferred by a mutation in Drosophila $GABA_A$ receptor of a residue that we have shown is exposed in the channel supports the interpretation that picrotoxin binds in the channel.

The interaction between the picrotoxin binding site and GABA binding is complicated. With the use of 15-min incubations, the presence or absence of GABA did not affect the binding of [^{3}H]dihydropicrotoxinin to rat brain membranes (Ticku et al., 1978). In electrophysiologic experiments, the binding of picrotoxin is use-dependent, i.e., picrotoxin binds more rapidly to the GABA-bound open state than to the closed state (Smart and Constanti, 1986; Inoue and Akaike, 1988; Twyman et al., 1989; Newland and Cull-Candy, 1992; Yoon et al., 1993); however, it does bind to the closed state of the receptor, although at a much slower rate (Newland and Cull-Candy, 1992). The binding of [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S]-TBPS) to $GABA_A$ receptors is competitively blocked by picrotoxin, is anion-dependent, and is inhibited by GABA (Squires et al., 1983). This suggests that the TBPS binding site partially overlaps the picrotoxin site, but TBPS must interact with other residues that undergo a conformational change following GABA binding. Similarly, the binding site of the γ-butyrolactones used in this study presumably partially overlaps the picrotoxin and TBPS sites. As with TBPS, the inhibitory effects of βEMGBL are inhibited by GABA (Yoon et al., 1993). Thus, the binding of GABA must alter the structure of the $GABA_A$ receptor in the region surrounding the picrotoxin binding site, both to account for the use-dependence of picrotoxin-induced block and to account for the inhibition of TBPS and βEMGBL binding. We have shown that the accessibility of α_1T261C is different in the presence and absence of GABA. This implies that GABA binding induces a conformational change in the channel lining in the region of 257–261. These GABA-induced changes may account for the use-dependence of picrotoxin block and the inhibition of TBPS binding by GABA.

Although all of the above results are compatible with the picrotoxin binding site being in the channel at the level of α_1Val257, the results above could also be interpreted with an allosteric model assuming close coupling between a distant picrotoxin binding site and the region of the channel that we have studied. In view of the allosteric coupling between various other $GABA_A$ receptor modulators such as benzodiazepines, barbiturates and neurosteroids, we cannot at present rule out the possibility that picrotoxin blocks conduction by an allosteric mechanism, although it seems less likely. If picrotoxin were to act by an allosteric mechanism, it could not act by simply locking the receptor in the closed state; in the closed state α_1V257C was accessible to $pCMBS^-$, but in the picrotoxin-blocked state the sulfhydryl at position α_1Val257 was inaccessible to $pCMBS^-$.

CONCLUSIONS

In conclusion, the structure of the $GABA_A$ receptor channel in the region of residues $\alpha_1$257 to $\alpha_1$261 appears to be important for several aspects of channel function. We have identified α_1Val257 and α_1Thr261, two residues in this region that line the channel of the $GABA_A$ receptor. The channel lining undergoes a structural change during gating, but the gate is at least as cytoplasmic as α_1Val257. The charge selectivity filter appears to be between positions 261 and 257 because the positively charged sulfhydryl reagent reacted with a sulfhydryl at the level of α_1Thr261 but not at the level of α_1Val257. Furthermore, we have shown that picrotoxin block of GABA-induced currents may result from picrotoxin binding in the channel lumen at the level of α_1Val257.

We thank Drs. Peter Seeburg and Allan Tobin for the $GABA_A$ subunit cDNAs, Drs. Jonathan Javitch and Arthur Karlin for advice and comments on this manuscript, Gilda Salazar-Jimenez and Celeste DeMarco for technical assistance, and Alex Fariborzian for synthesizing $MTSEA^+$.

This work was supported in part by National Institutes of Health National Institute of Neurological Disorders and Stroke Grants NS30808 (Myles H. Akabas) and NS14834 (Douglas F. Covey) and an American Heart Association Grant-in-Aid to Myles H. Akabas. Myles H. Akabas is the recipient of a Klingenstein Award in Neuroscience and is an Established Scientist of the New York Heart Association. Ming Xu was supported in part by National Institutes of Health Training Grant NS07258.

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